

**Impacts of the human pharmaceutical diclofenac in the
aquatic environment**

Submitted by Alvine Coralie Mehinto

To the University of Exeter as a thesis for the degree of Doctor of Philosophy in
Biological Sciences, October 2009

This thesis is available for Library use on the understanding that it is copyright material
and that no quotation from the thesis may be published without proper
acknowledgment.

I certify that all material in this thesis which is not my own work has been identified and
that no material has previously been published and approved for the award of a degree
by this or any other University.

Signature ...A. MEHINTO....

Abstract

An increasing number of pharmaceuticals have been found in the aquatic environment and the issue has become a human and environmental health concern. Many pharmaceuticals are not fully degraded in wastewater treatment plants (WWTPs) and are continuously released in the aquatic environment resulting in concentrations in the low $\mu\text{g/l}$ range in the receiving waters. Diclofenac is a widely used non-steroidal anti-inflammatory drug (NSAID) and is persistent in the aquatic environment. This pharmaceutical has been frequently reported in wastewater effluents, surface waters, groundwaters and even drinking water. NSAIDs are known to inhibit the cyclooxygenase activity, an enzyme present in many species of the animal kingdom responsible for the synthesis of prostanoids, and chronic exposure to environmental diclofenac may have detrimental effects on metabolism of non-target organisms including microbes and fish. In this thesis, microbiology, genomics and metabolomics approaches were used to investigate the effects of diclofenac on aquatic microbes and fish.

In the first study of the thesis (chapter 3), the biodegradation of selected NSAIDs was investigated, together with their potential toxicity to aquatic microbes. Aerobic biodegradation experiments were conducted using activated sludge and wastewater effluents as microbial inocula and diclofenac, ketoprofen or naproxen as sole carbon source (1-10 mg/l) in order to isolate and identify the bacterial degraders. Changes in the bacterial populations were monitored by optical density and PCR-DGGE. The analytical techniques solid phase extraction (SPE) and ultraperformance liquid chromatography-mass spectrometry (UPLC-TOF-MS) were optimised to quantify the pharmaceuticals in environmental samples. High recovery rates were obtained with 94% for diclofenac; 92% for ketoprofen and 85% for naproxen and with detection capabilities down to 3-7 ng/l. Results from the biodegradation experiments showed that ketoprofen and naproxen were eliminated at up to 99 and 55% respectively over a 40 days period. Consistently with previous studies, diclofenac showed no significant degradation. In all the enrichments, a significant decrease in the bacterial abundance was observed as a consequence of NSAIDs exposure and attempts to isolate the bacterial degrading populations were unsuccessful. Given the apparent micro-toxicity of these NSAIDs, the standardised test Microtox[®] was carried out with *Vibrio fischeri*. The EC₅₀ (15 min) estimated ranged from 13.5 mg/l \pm 2.3 for diclofenac to 42.1 mg/l \pm 3.9 for naproxen. Further toxicological tests were performed with diclofenac on bacterial strains isolated from activated sludge. Growth inhibitory effects were observed from 50-70 mg/l for *Micrococcus luteus*, *Zoogloea ramigera* and *Comamonas denitrificans*. *Pseudomonas putida* seemed more tolerant to diclofenac exposure and toxic effects were observed from 90 mg/l. These studies showed that diclofenac was the most toxic NSAID but toxicological effects in bacteria only occurred at concentrations at least 1,000 times higher than those found in the environment. However, chronic exposure to lower concentrations may cause similar interferences and affect the degradation potential of naturally occurring microbial populations.

The second study (chapter 4) investigated the biological effects of sub-chronic exposure to waterborne diclofenac (0.5, 1, 5 and 25 $\mu\text{g/l}$) in female juvenile rainbow trout *Oncorhynchus mykiss*. After 21-day exposure, mRNA expression levels of cytochrome p450 1a1 (*cyp1a1*), cyclooxygenase (*cox*) 1 and 2, and *p53* were investigated in the liver, kidney and gills using RT-PCR and QPCR. These genes were selected as they are likely targets for diclofenac in mammals. Histopathological investigations were carried out in the small intestine, liver and kidney because

diclofenac has been reported to induce toxicity responses in these tissues. Fish bile was also analysed by SPE and UPLC-TOF-MS to evaluate the bioconcentration potential of diclofenac and look for evidences of diclofenac metabolism. Results showed a significant reduction of both *cox1* and *cox2* expression in the liver, gills and kidney from 1 µg diclofenac/l. In contrast diclofenac induced an increase in mRNA levels for *cyp1a1* in the liver and gills but a significant reduction of *cyp1a1* expression in the kidney from 1 µg/l. There were no clear effects of diclofenac on the mRNA levels of *p53*. Diclofenac exposure caused tissue damages at exposure concentrations as low as 1 µg/l. Histopathological injuries included inflammation, hyperplasia and fusion of the villi in the small intestine and tubule necrosis in the kidney. There were no obvious changes in the liver of diclofenac-exposed fish. The analysis of bile revealed a bioconcentration potential between 509 ± 27 and 657 ± 25 . A reactive metabolite of diclofenac was also detected at the highest exposure concentration which may be responsible for the severe injuries found in those fish. Sub-chronic exposure to environmental concentrations of diclofenac altered gene expression and it is possible that long term exposure to environmental diclofenac lead to significant impacts on fish health.

In the final part of this thesis (chapters 5 and 6) effects on the metabolite composition of biofluids were analysed in diclofenac-exposed fish. This work entailed developing and validating appropriate methodologies to analyse fish bile and blood plasma. Methanol extraction and UPLC-TOF-MS were optimised to analyse the plasma metabolome but the methodologies were not suitable to detect low abundance molecules such as eicosanoids due to the interferences (ion suppression) in the samples matrix. Multivariate data analysis failed to detect the endogenous metabolites of the plasma affected by the chemical exposure. The only discriminating metabolite was found after analysis of the plasma samples from control vs. 25 µg/l treatment groups and identified as the exogenous compound diclofenac. To analyse the bile, the developed SPE methodology was carried out in order to separate the metabolites between a free steroids (fatty acids, eicosanoids, etc.) fraction and a conjugated steroids (bile salts) fraction. Due to high levels of taurocholic acid masking other metabolites in the conjugated fraction, some bile samples were hydrolysed to deconjugate these metabolites. The non-hydrolysed and hydrolysed bile fractions were analysed by UPLC-TOF-MS in positive and negative ionization. Multivariate data analysis using principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) revealed significant perturbations in the bile metabolite profile of diclofenac-exposed rainbow from the lowest exposure concentration (0.5 µg/l). Over 50 metabolites were elevated or reduced as a result of the 21-day exposure, suggesting that diclofenac affected several metabolic pathways. One metabolite was identified as a lipooxygenase product. This suggests that the inhibition of prostanoids synthesis can cause a shift in the arachidonic cascade and increase the synthesis of other eicosanoids. Most of the other discriminative metabolites remain unidentified and FT-MS analysis will be performed to obtain a structural identity. The metabolomics study further highlights the concern of environmental diclofenac in non-target organisms and the need to investigate the metabolic pathways affected.

Acknowledgements

First of all I would like to thank my supervisors Professor Charles Tyler, Dr Sara Burton and Professor Hilary Lappin-Scott for their support and encouragement throughout this study. I am eternally grateful to my unofficial supervisor Dr Elizabeth Hill from University of Sussex for teaching me metabolomics and for her invaluable help with the chemical analyses.

Special thanks to Peter Splatt and Jan Shears for the vast technical support. I would like to thank Morley Williams from Southwest Water from (Countess Wear) for its help during sampling. I would also like to thank Dr Eduarda Santos, Dr Amy Filby, Dr Patrick Hamilton, Dr Lisa Bickley, and Dr Anke Lange for their precious help in the lab and during the writing process. I am very grateful to all the members of the former EMERGE group and the EMFB group at University of Exeter and my colleagues at University of Sussex for their friendship and support. Special mentions must be made to Viv, Rhys, Tess, Jenny and Luanne who helped me during the fish exposure but also Rachel, Max, Theresa, Laura and Okhyun. Big thank you to Dalia and Nicola for their precious advice to “carry on regardless” and support. A huge thank you to Elena for guiding me through the metabolomics, and being such a good friend.

Thank you to Marco, Hervé, tante Colette, tante Arlette, my family and friends who have supported me during the PhD. Special thank you to my cousin Dr Bobo for everything...

Finally a huge thank you to my parents for their unconditional love, patience, support and believing in me.

This thesis is dedicated to my aunts Madina Kelani (1952-2009), Yvette Paraiso (1963-2008) and my cousin Titi Bouraima (1969-2008).

Table of contents

Title page and declaration	i
Abstract	ii
Acknowledgements	iv
Table of contents	v
List of figures	xii
List of tables	xv
List of abbreviations	xvii
List of abbreviated chemicals	xx

Chapter 1: General Introduction

1.1. Pharmaceuticals in the environment.....	1
1.1.1. Origin.....	2
1.1.2. Occurrence in the aquatic environment.....	4
1.2. Non-steroidal anti-inflammatory drugs.....	7
1.2.1. Mode of action.....	9
1.3. Removal of NSAIDs in the environment	11
1.3.1. Wastewater treatment plants.....	11
1.3.2. Removal efficiency in wastewater treatment plants.....	13
1.3.3. Biodegradation	13
1.3.4. Sorption	15
1.3.5. Abiotic processes.....	16
1.4. Environmental impact of NSAIDs	17
1.4.1. Acute toxicity	17
1.4.2. Chronic toxicity	18
1.5. Xenobiotic metabolism in fish	20

1.5.1. Cytochrome P450 monooxygenase system	20
1.5.2. Cytochromes P450 in fish and use as biomarker of aquatic pollution	21
1.5.3. Bile as major excretory route of pharmaceuticals	22
1.6. Analysis of pharmaceuticals in environmental samples.....	23
1.6.1. Sample preparation.....	23
1.6.2. Detection techniques	24
1.7. Ecotoxicogenomics to assess biological effects of pharmaceuticals in aquatic organisms.....	25
1.7.1. Genomics.....	25
1.7.2. Proteomics	26
1.7.3. Metabolomics	27
1.8. Bioinformatics	28
1.9. Aims of the thesis	29

Chapter 2: General Materials and Methods

2.1. Biodegradation studies	33
2.1.1. Sampling sites.....	33
2.1.2. Bacterial growth media.....	33
2.1.3. Microcosm enrichment cultures	35
2.1.4. Sub-culturing method.....	36
2.2. Nucleic acid extraction	36
2.2.1. 5% CTAB/phosphate buffer	36
2.2.2. Extraction procedure	36
2.3. Agarose gel electrophoresis.....	37
2.4. Polymerase chain reaction amplification of 16S rRNA gene.....	38
2.5. Microtox acute toxicity test.....	39
2.6. Disc diffusion assay.....	39
2.6.1. Bacterial strains	39
2.6.2. Toxicity test protocol.....	40

2.7. Detection of pharmaceuticals in water samples	40
2.7.1. Solid phase extraction (SPE).....	40
2.7.2. Ultraperformance liquid chromatography time-of-flight mass spectrometry ...	41
2.7.3. Quantification of pharmaceuticals.....	43
2.8. Fish studies	44
2.8.1. Supply and maintenance of the fish.....	44
2.8.2. Experimental set up	44
2.8.3. Dissection and tissue collection.....	46
2.9. Total RNA extraction and quantification	47
2.10. Reverse transcription (RT)-PCR	48
2.11. Quantitative real-time PCR (Q-PCR).....	49
2.11.1. Primer design for Q-PCR	49
2.11.2. Optimisation of primer-pair annealing temperature	50
2.11.3. Determination of Q-PCR amplification efficiency and melt curve.....	50
2.11.4. QPCR protocol for tissues	51
2.11.5. Data analysis.....	53
2.12. Histopathology on fish tissues.....	54
2.12.1. Dehydration and embedding.....	54
2.12.2. Sectioning of the embedded tissues.....	54
2.12.3. Haematoxylin and Eosin (H&E) Staining	55
2.12.4. Analysis of fixed tissue sections.....	55
2.13. Metabolomic studies.....	58
2.13.1. Hydrolysis of bile samples	58
2.13.2. Solid phase extraction of bile samples	59
2.13.3. Methanol extraction of blood plasma samples	59
2.13.4. Pre-processing of data	60
2.13.5. Multivariate analysis	60
2.13.6. Model and data validation	61

Chapter 3: Biodegradation of non-steroidal pharmaceuticals in the aquatic environment and their toxicity to microbes

Contribution of each author.....	63
Abstract	64
3.1. Introduction	65
3.2. Materials and methods.....	68
3.2.1. Chemicals and reagents	68
3.2.2. Environmental sampling.....	68
3.2.3. Biodegradation study.....	68
3.2.4. Solid Phase Extraction (SPE).....	70
3.2.5. Ultraperformance Liquid Chromatography/Electrospray ionization time-of-flight- Mass Spectrometry	71
3.2.6. Quantification of NSAIDs.....	73
3.2.7. Bioluminescence assay.....	73
3.2.8. Disc diffusion assay.....	74
3.2.9. Predicted pathway for the biodegradation of diclofenac	74
3.3. Results	74
3.3.1. Recovery efficiencies for NSAIDs.....	74
3.3.2. Biodegradation study.....	75
3.3.3. Diclofenac degradation study.....	77
3.3.4. Bioluminescence assay.....	77
3.3.5. Disc diffusion assay.....	80
3.6. Prediction of the biodegradation pathway for diclofenac.....	80
3.4. Discussion	82
3.5. Conclusions	88

Chapter 4: Uptake and biological effects of environmentally relevant concentrations of the non-steroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*)

Contribution of each author.....	90
----------------------------------	----

Abstract	91
4.1. Introduction	92
4.2. Materials and methods.....	94
4.2.1. Diclofenac exposure	94
4.2.2. Fish sampling.....	94
4.2.3. Condition factor.....	95
4.2.4. Analysis of diclofenac in water and bile samples	95
4.2.5. RNA extraction and reverse transcription (RT) PCR.....	96
4.2.6. Primer design and real-time PCR optimisation.....	96
4.2.7. Real-time PCR.....	97
4.2.8. Histology	98
4.2.9. Data analysis.....	98
4.3. Results	99
4.3.1. Analysis of diclofenac concentrations in the tank water	99
4.3.2. Condition factor.....	99
4.3.3. Concentration of diclofenac and identification of its metabolites in fish bile...99	
4.3.4. Target gene expression.....	102
4.3.5. Histopathological findings.....	105
4.4. Discussion	110
Supplementary materials	115

Chapter 5: Development of analytical techniques for metabolic profiling of biofluids in rainbow trout (*O. mykiss*)

5.1. Introduction	118
5.2. Materials and methods.....	120
5.2.1. Chemicals	120
5.2.2. Sample collection	120
5.2.3. Preparation of bile samples	122
5.2.4. SPE fractionation and recovery of standard metabolites.....	123

5.2.5. Preparation of plasma samples	123
5.2.6. UPLC-TOF-MS analysis	123
5.2.7. Data handling.....	124
5.2.8. Multivariate data analysis of plasma samples	125
5.3. Results	125
5.3.1. Method development for metabolic profiling of bile	125
5.3.1.1. Analysis of the chromatograms for the bile extracts	125
5.3.1.2. Quantitative analysis of the bile extracts.....	128
5.3.2. SPE fractionation and recovery of standard metabolites.....	132
5.3.3. Method development for metabolic profiling of plasma samples	135
5.3.4. PCA and PLS-DA analyses of plasma samples from diclofenac exposure....	140
5.3.5. Identification of class-separating markers.....	144
5.4. Discussion	144
5.5. Conclusions	149

Chapter 6: Identifying the biological effects of diclofenac in fish using metabolomics profiling

6.1. Introduction	150
6.2. Materials and Methods	152
6.2.1. Chemicals	152
6.2.2. Diclofenac exposure and sample collection	152
6.2.3. Bile hydrolysis.....	153
6.2.4. Metabolite extraction and UPLC-TOF-MS analysis.....	153
6.2.5. Data transformation, pre-processing and pre-treatment	154
6.2.6. Multivariate data analysis.....	155
6.2.7. Identification of metabolites.....	155
6.3. Results	156
6.3.1. PCA overview of the free metabolites and conjugated metabolites fractions from non-hydrolysed bile in control and diclofenac-exposed fish.....	156

6.3.2. Overview of PLS-DA and OPLS models for non-hydrolysed bile	157
6.3.3. PCA overview of the free metabolites (EA+FA) fraction from hydrolysed bile extracts.....	161
6.3.4. Overview of PLS-DA and OPLS models for hydrolysed bile extracts.....	161
6.3.5. Identity of metabolites of diclofenac.....	165
6.3.6. Identity of metabolite markers of diclofenac exposure	168
6.4. Discussion	174
5. Conclusions	180
 Chapter 7: General Discussion	
Discussion	181
Future research	189
Conclusions	191
 References	 193

List of figures

Figure 1.1: Routes of entry for pharmaceuticals.....	3
Figure 1.2: Chemical structure of selected NSAIDs.....	8
Figure 1.3: Schematic diagram of arachidonic acid conversion to prostanoids.....	10
Figure 1.4: Schematic diagram of a wastewater treatment plant.....	12
Figure 1.5: Hydroxylation reactions catalysed by cytochrome P450s.....	21
Figure 2.1: Maps of the sampling sites.....	34
Figure 2.2: Diclofenac <i>in vivo</i> experimental set-up.....	45
Figure 2.3: Real-time PCR amplification graph.....	52
Figure 2.4: Melt curve analysis.....	52
Figure 3.1: Solid phase extraction protocol at acidic pH using Oasis HLB 6cc cartridges.....	72
Figure 3.2: Degradation of NSAIDs (conc. 10 mg/l) with sludge samples incubated in orbital shaker at 25 °C.....	76
Figure 3.3: Degradation of diclofenac (1 mg/l) by environmental samples.....	78
Figure 3.4: Proposed degradation pathways of diclofenac using UMBBD.....	83
Figure 4.1: Average threshold cycle (C_T) for <i>rpl8</i> amplification in liver, gills and kidney after 21 day exposure to diclofenac.....	103
Figure 4.2: Relative expression of <i>cox1</i> (a), <i>cox2</i> (b), <i>cyp1a1</i> (c) and <i>p53</i> (d) in rainbow trout after 21-day diclofenac exposure.....	104
Figure 4.3a: Histopathological lesions in small intestine of rainbow trout induced by diclofenac exposure.....	106
Figure 4.3b: Semi-quantitative assessment of histopathological lesions in the small intestine of diclofenac-exposed rainbow trout.....	107
Figure 4.4a: Histopathological lesions in the kidney of diclofenac-exposed rainbow trout after 21 days.....	108

Figure 4.4b: Semi-quantitative assessment of histopathological lesions in the kidney of diclofenac-exposed rainbow trout.....	109
Figure S4.1: Primers designed and optimised for QPCR assay.....	115
Figure S4.2: Condition factor of rainbow trout.....	116
Figure S4.3: Histological sections of liver tissue from control and diclofenac treated rainbow trout.....	117
Figure 5.1: Total ion chromatograms (as base peak intensity BPI) of untreated bile in +ESI and –ESI modes.....	126
Figure 5.2: Spectral ion chromatograms of the saturated peaks in untreated bile in +ESI and –ESI modes.....	126
Figure 5.3: Total ion chromatograms (as base peak intensity BPI) of fractionated non-hydrolysed bile and blank samples in –ESI mode.....	127
Figure 5.4: Total ion chromatograms (as base peak intensity BPI) of fractionated hydrolysed bile and blank samples in –ESI mode.....	129
Figure 5.5: Chromatograms of target metabolites and the internal standards.....	133
Figure 5.6: Total ion current (as base peak intensity BPI) of plasma sample from control fish in +ESI (a) and –ESI mode (b).....	137
Figure 5.7: Chromatograms of eicosanoids standards (4 pg/μl) in water samples run in –ESI mode with acidic mobile phase.....	138
Figure 5.8: Selected ion chromatograms (-ESI) of the eicosanoids recovered in the 80% methanol extracts of spiked plasma samples.....	141
Figure 5.9: Score plots from partial least squares discriminant analysis (PLS-DA) of rainbow trout plasma following diclofenac exposure.....	143
Figure 5.10: Mass spectra of diclofenac ion (<i>m/z</i> 294.008) in –ESI mode in plasma.....	145
Figure 6.1: PLS-DA score plots using the first 2 components of the non-hydrolysed bile fractions of control fish (■) and fish exposed to 25 μg diclofenac/l (●).....	160
Figure 6.2: Score plots of the multivariate discriminant analysis of the free metabolites (EA+FA) fraction for hydrolysed bile from diclofenac exposure.....	164

Figure 6.3: a) S-plot from the OPLS analysis of hydrolysed bile of control and 5 µg/l groups in the free metabolites fraction analysed in –ESI mode. b) trend view of some of the markers of diclofenac exposure identified by OPLS analysis of control and 5 µg/l exposed trout.....	166
Figure 6.4: Total ion chromatograms showing diclofenac and its metabolites in diclofenac-exposed rainbow trout.....	169
Figure 6.5: Spectral ion chromatograms of diclofenac and hydroxydiclofenac fragments produced by UPLC-TOF-MS analysis.....	169
Figure 6.6: Conversion of arachidonic acid into eicosanoids.....	179

List of tables

Table 1.1: Concentrations of selected pharmaceuticals in the aquatic environment.....	6
Table 2.1: Solid phase extraction protocol.....	42
Table 2.2: Processing program for histological analyses.....	56
Table 2.3: H&E staining protocol for fixed sections.....	57
Table 3.1: Properties of NSAIDs used in this study.....	69
Table 3.2: Acute toxicity (EC ₅₀ , 15 min) of NSAIDs using <i>Vibrio fischeri</i>	79
Table 3.3: Mean diameter of the inhibition zone.....	81
Table 4.1: Mean measured diclofenac concentrations ($\mu\text{g/l} \pm \text{SE}$) in replicate tanks water.....	100
Table 4.2: Measured diclofenac concentrations (ng/ml, mean \pm SE) and estimated bioconcentration in rainbow trout bile after 21-day exposure.....	100
Table 4.3: Putative metabolites of diclofenac identified in fish bile.....	101
Table S4.1: Primers designed and optimised for real-time PCR analysis.....	116
Table 5.1: Preparation of standard stock solutions.....	121
Table 5.2: Number of markers estimated by MarkerLynx for non-hydrolysed bile samples untreated and fractionated using SPE.....	130
Table 5.3: Number of markers estimated by MarkerLynx for hydrolysed bile samples fractionated using SPE.....	131
Table 5.4: UPLC-TOF-MS analysis of target metabolites in aqueous samples.....	134
Table 5.5: Number of markers detected after methanol extraction of plasma samples.....	136
Table 5.6: Limit of detection (LOD) of eicosanoids in water and plasma samples using UPLC-TOF-MS.....	139
Table 5.7: Performance parameters of multivariate discriminant models for the comparison of control and diclofenac exposed rainbow trout.....	142

Table 6.1: Performance parameters of principal component analyses for the comparison of the metabolic profiles in non-hydrolysed bile extracts.....	158
Table 6.2: Multivariate discriminant models for non-hydrolysed bile samples of control and diclofenac-exposed rainbow trout.....	159
Table 6.3: Performance parameters of principal component analyses for the comparison of the metabolic profiles in hydrolysed bile extracts.....	162
Table 6.4: Multivariate discriminant models for the comparison of control and diclofenac exposed rainbow trout in hydrolysed bile.....	163
Table 6.5: Identification of diclofenac and putative metabolites.....	167
Table 6.6a: Markers of diclofenac exposure identified in non-hydrolysed bile of rainbow trout.....	170
Table 6.6b: Markers of diclofenac exposure identified in hydrolysed bile of rainbow trout.....	170-172

List of abbreviations

2D-PAGE	2-dimensional polyacrylamide gel electrophoresis
AHH	aryl hydrocarbon hydroxylase
ANOVA	analysis of variance
BLAST	Basic Local Alignment Search Tool
BC	bioconcentration
bp	base pair
BPI	base peak intensity
CAS	chemical abstract service
cDNA	complementary deoxyribonucleic acid
CE-MS	capillary electrophoresis - mass spectrometry
cm	centimetre
<i>cox</i>	cyclooxygenase
C_T	threshold cycle
<i>cyp</i>	cytochrome P450
<i>cyp1a1</i>	cytochrome P450 1a
Da	dalton
dATP	deoxyadenosine triphosphate
DCF	diclofenac
dCTP	deoxycytidine
dGTP	deoxyguanosine triphosphate
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTPs	deoxyribonucleotide triphosphates
dTTP	thymidine triphosphate
E_2	17 β -estradiol
EC_{50}	50% effect concentration
ECOSAR	Ecotoxicological Structure Activity Relationship
EDC	endocrine disrupting chemical
EE_2	17 α -ethinylestradiol
EPA	eicosapentaenoic acid
EROD	ethoxyresorufin- <i>O</i> -deethylase
ESI	electrospray ionisation
eV	electron volt
FT-MS	Fourier transform - mass spectrometry
g	gram
GC	gas chromatography
HEPES	hydroxyeicosapentaenoic acids
HETES	hydroxyeicosatetraenoic acids
HPLC	high performance liquid chromatography
hr	hour
IMS	industrial methylated spirit

IS	internal standard
Kg	kilogram
kV	kilo volt
l	litre
LC	liquid chromatography
LOD	limit of detection
LOEC	lowest observed effect concentration
M	mole per litre
M+H	protonated molecule
M-H	deprotonated molecule
MEC	measured environmental concentration
mg	milligram
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
mm	millimetre
mM	millimole per litre
M-MLV	moloney murine leukaemia virus
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSM	minimal salts medium
MW	molecular weight
<i>m/z</i>	mass to charge ratio
n	number of samples
ng	nanogram
nm	nanometre
nM	nanomole per litre
NMR	nuclear magnetic resonance
NSAIDs	non steroidal anti-inflammatory drugs
OD	optical density
OPLS	orthogonal partial least squares projection to latent structures
p	statistical probability
P450	cytochrome P450
<i>p53</i>	tumour protein 53
PAH	polycyclic aromatic hydrocarbon
PCA	principal component analysis
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PEC	predicted environmental concentration
pg	picogram
PIT	passive integrated transponder
PLS-DA	partial least squares projection to latent structures discriminant analysis
pmol	picomole
PNEC	predicted no effect concentration

ppm	part per million
Q^2	cumulative variation predicted by the PCA or PLS model
Q-PCR	real-time quantitative reverse transcription PCR
R^2	variation explained by the PCA or PLS model
RNA	ribonucleic acid
Rnase	ribonuclease
<i>rpl8</i>	ribosomal protein l8
rpm	rotation per minute
rRNA	ribosomal ribonucleic acid
RT	retention time
RT-PCR	reverse transcription polymerase chain reaction
SE	standard error
sec	seconds
SPE	solid phase extraction
TIC	total ion chromatogram
T_M	melting temperature
TOF	time of flight
UPLC-MS	ultraperformance-liquid chromatography - mass spectrometry
UV	ultraviolet
v/v	volume to volume ratio
V	volt
vs.	versus
WWTP	wastewater treatment plant
$^{\circ}\text{C}$	degree Celsius
μg	microgram
μl	microlitre
μm	micrometre
μM	micromole per litre

List of abbreviated chemicals

11-HETE	±11-hydroxyeicosatetraenoic acid
CoCl ₂	phosgene
CTAB	cetyltrimethylammonium bromide
CuSO ₄	copper (II) sulphate
E ₂ - <i>d4</i> -S	[2,4,16,16- <i>d4</i>] 17β-estradiol sodium 3-sulfate
EDTA	ethylenedinitrilotetraacetic acid
H ₂ SO ₄	sulphuric acid
K ₂ HPO ₄	potassium phosphate dibasic
KH ₂ PO ₄	potassium dihydrogen phosphate
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
MnSO ₄	manganese sulphate
NaCl	sodium chloride
NaOH	sodium hydroxide
Na ₂ SO ₄	sodium sulphate
NH ₄ OH	ammonium hydroxide
(NH ₄) ₂ SO ₄	ammonium sulphate
P- <i>d9</i>	[2,2,4,6,6,17α-21,21,21- <i>d9</i>] progesterone
PGB ₂	prostaglandin B ₂
PGE ₂	prostaglandin E ₂
PGJ ₂	prostaglandin J ₂
TAE	Tris-acetate-EDTA
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
TxB ₂	tromboxane B ₂
ZnSO ₄	zinc sulphate